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Research Article

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Reproductive innovation and the recognition of a new genus within the Polystomatidae (Monogenea) infecting chelonian vertebrates

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Abstract: Polystomatid monogeneans have a wide diversity of life cycles correlated with the varied ecology and behaviour of their aquatic vertebrate hosts. Typically, transmission involves a swimming infective larva but most hosts are amphibious and invasion is interrupted when hosts leave water. A key life cycle adaptation involves a uterus that, in the most specialised cases, may contain several hundred fully-developed larvae prepared for instant host-to-host transmission. By contrast, one subfamily of the Polystomatidae – the Polystomoidinae, specific to chelonians (freshwater turtles) – has a simplified reproductive system without a uterus. Recently, *Polystomoides nelsoni* Du Preez et Van Rooyen, 2015 has been described with a uterus containing multiple eggs. The present study explores the exceptional interest of this parasite – for the functional biology of egg production, for the evolution of a reproductive system unique amongst *ca* 60 species in the subfamily, and for systematic relationships. A new genus is proposed, *Uteropolystomoides* gen. n., separate from the four currently-recognised genera *Polystomoides* Ward, 1917, *Uropolystomoides* Tinsley et Tinsley, 2016, *Neopolystoma* Price, 1939 and *Polystomoidella* Price, 1939 which lack a uterus. In addition, *U. nelsoni* (Du Preez et Van Rooyen, 2015) comb. n. has a suite of distinctive copulatory structures: a massive genital bulb with an exceptionally large number of very long genital spines and hyper-development of the vaginal openings. These characters set *U. nelsoni* apart from all other polystomoidines worldwide except *Polystomoides multifalx* Stunkard, 1924 and *P. stunkardi* Harwood, 1931. Missing data for these latter species preclude definitive assessment of inter-relationships but the distinguishing characters of *U. nelsoni*, especially the unique occurrence of the uterus, suggest a novel evolutionary pathway isolated from other lineages of polystomatids infecting chelonians.

Keywords: *Polystomoides*, *Uteropolystomoides nelsoni*, reproduction, evolution, uterus, oötype, helminth egg production, key

Monogeneans of the family Polystomatidae Gamble, 1896 are remarkable for their evolutionary diversification in parallel with their freshwater vertebrate hosts. Lineages infect a lungfish, all groups of amphibians (caecilians, anurans and urodeles), one group of reptiles (chelonians) and one mammal (the hippopotamus). The phylogeny of these lineages has been linked to the early evolution of tetrapods: molecular analyses have suggested an origin around 425 million years ago and subsequent major divergences have been calibrated with the chronology of plate tectonic events (Verneau et al. 2002, Badets et al. 2011, Héritier et al. 2015).

Alongside this record of parasite phylogeny over a long period of evolutionary time, the Polystomatidae is also remarkable for its diversity of life cycle patterns which are amongst the most varied in the Platyhelminthes (Tinsley 1993). Transmission, involving an aquatic infective larva, is strictly limited to the occurrence of the host in water. The wide range of hosts includes some that are more or less continuously aquatic and therefore potentially exposed to uninterrupted invasion; others are terrestrial for varying

periods in their lives, restricting parasite transfer to sometimes very brief visits to water. Transmission strategies are most varied in the lineages of polystomatids that infect anuran amphibians where life cycles can be completed in such hostile conditions as mountain torrents, floodwaters of tropical forests and ephemeral ponds in deserts (Tinsley 1983, 1990, 2005).

The key to the success of polystomatids in exploiting brief opportunities for host-to-host transfer lies, fundamentally, in the structure and function of the uterus. In its simplest form, the uterus provides a conduit to the outside for eggs assembled in the oötype. This basic tube may also permit storage of eggs after assembly and hence the possibility that release to the exterior may be controlled. An extreme development of this potential is illustrated by *Pseudodiplorchis americanus* (Rodgers et Kuntz, 1940), a parasite of the desert toad *Scaphiopus couchii* Baird, where the entire annual reproductive output may be stored in preparation for mass release during a few hours each year when hosts enter temporary ponds to spawn (Tinsley 1999).

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In this and over a dozen other polystomatid genera infecting anurans, the uterus is greatly enlarged, typically extending throughout the central body space and able to accommodate several hundred egg capsules (Table 1). Other internal organs – ovary, testes, vaginae, vitellarium and intestine – are typically confined laterally and posteriorly in a wide range of different configurations (Tinsley 1983).

In complete contrast, the second largest group within the Polystomatidae – the Polystomoidinae Yamaguti, 1963, containing all the species that are specific to chelonians – is morphologically highly uniform. All species, currently over 60 assigned to four genera, have a simplified reproductive system with only minor variations in the arrangement of internal organs. For the past 100 years (since Ward 1917), it has been considered that the major unifying characteristic across the chelonian polystomatids is the lack of a uterus: only a single egg is present at any one time, held within the chamber in which encapsulation occurs – the oötype.

However, the most recently described species, *Polystomoides nelsoni* Du Preez et Van Rooyen, 2015, a parasite of the North American turtle *Pseudemys nelsoni* Carr, overturns the apparent uniformity within the subfamily. Du Preez and Van Rooyen (2015) reported that, in their sample of ten specimens, *P. nelsoni* has a uterus containing up to eight eggs. This new species was distinguished by characters including marginal hooklet morphology, body length and haptor dimensions but the authors did not comment further on the presence of a uterus nor include it as a distinguishing character. The possession of a uterus sets *P. nelsoni* apart from all other known polystomatids infecting chelonians. It opens up the potential for life cycle adaptations equivalent to those of polystomatids in anuran amphibians and it raises questions about the origins and relationships of its unique reproductive organisation. The aim of the present study is first, to consider the significance of the presence or absence of a uterus for life cycle biology, and second, to assess implications for the evolution and systematics of this lineage within the Polystomatidae.

MATERIALS AND METHODS

Microscope studies were based on the paratype series of *Polystomoides nelsoni*, comprising nine whole mount stained specimens, kindly loaned by Louis Du Preez and the National Museum, Bloemfontein, and on comparative material in the author's collection of the four genera currently recognised in the Polystomoidinae. Studies were not intended to redescribe the species: the published account of morphology and measurements (Du Preez and Van Rooyen 2015) included meristic data for the holotype, not examined here, and had the advantage of living material of adults and oncomiracidia that would have provided a clearer view of internal structure and organisation. Instead, observations add to specific aspects of reproductive morphology relevant to interpretation of functional biology and relationships.

RESULTS

Morphology

The paratype specimens of *Polystomoides nelsoni* confirm that the ovary (or germarium), vitelline follicles, vaginae and their interconnecting ducts have a configuration that does not differ in principle from that typical of other Polystomoidinae. Several organs in these specimens are unusually small for polystomoidines: thus, ovary length and width are about equal to the dimensions of a single egg capsule. Using the mean measurements reported by Du Preez and Van Rooyen (2015), ovary length is 4% of worm body length (excluding the haptor); egg length is 4% and testis length is 7% of body length. The most distinctive characteristics of this taxon concern the distal parts of the reproductive tract.

Vaginae are not mentioned in the species description (although they are depicted in the holotype illustration): the whole mount specimens show that the size and structure of their openings is remarkable amongst species of *Polystomoides* Ward, 1917. On each side of the body, the vaginae open on a prominent, broadly oval, mound of densely-stained tissue. The anterior-posterior axis of this swelling is, on average, about 17% (max. 19%) of body length. In profile, the tumescence appears to be perforated by a series of channels converging on the vaginal duct opening, but this impression actually reflects that the mound is composed of a series of inwardly-directed finger-like lobes (with the intervening thinner tissues appearing as channels between more heavily-stained lobes).

The uterus is not depicted in the drawing of the holotype (Du Preez and Van Rooyen 2015) and the eggs are shown without any enclosing structure, but the species description refers to it as a 'short tubular uterus'. However, the paratype specimens demonstrate that the uterus forms a sac-like structure with a distinct wall surrounding the irregularly-arranged eggs (rather than a tube containing a linear series of eggs as in most polystomatids equipped with a uterus). Given the unique occurrence of a uterus in this species, further observations on living worms and histological sections are required. The holotype drawing shows this individual with five egg capsules. The nine paratype specimens include one pre-adult, with only early concentrations of cells developing in the ovary and testis and no egg capsule; the other paratypes contain 1, 1, 3, 3, 4, 6 and 8 egg capsules *in utero*.

The testis is a single, compact organ in the mid-body, typical of polystomoidines, with a vas deferens leading directly anteriorly. Du Preez and Van Rooyen (2015) reported that the genital atrium is armed with spines, but their description and measurements actually refer to the genital bulb not an 'atrium'. The genital bulb of the paratype specimens is massive, with a coronet comprising an exceptionally large number of very long spines: the species

Table 1. Variation in selected reproductive characters amongst polystomatid monogeneans.

Genus	Hosts	Ovary position	Uterus length	Uterus extent relative to ovary	Vaginae	Larval development within parent worm	Genital spines (number)	Reference
<i>Uteropolystomoides</i> gen. n.	chelonian	+	+	+	+	0	+++++	1
<i>Uropolystomoides</i> Tinsley et Tinsley, 2016	chelonian	+	0	-	+	+	++++	1
<i>Polystomoides</i> Ward, 1917	chelonian	+	0	-	+	0	+++	1
<i>Polystomoidella</i> Price, 1939	chelonian	+	0	-	+	++	++*	1
<i>Neopolystoma</i> Price, 1939	chelonian	+	0	-	+	0	++	1
<i>Protopolystoma</i> Bychowsky, 1957	anuran	+	0	-	0	0	+	2
<i>Polystoma</i> Zeder, 1800	anuran	+	+	+	+	+	+	2
<i>Metapolystoma</i> Combes, 1976	anuran	++	++	+	+	++	+	3
<i>Kankana</i> Raharivololoniaina, Verneau, Berthier, Vences et Du Preez, 2011	anuran	++	+++	++	0	++	+	4
<i>Eupolystoma</i> Kaw, 1950	anuran	+++	+++	+	+	++	+	5
<i>Pseudodiplorchis</i> Yamaguti, 1963	anuran	+	+++	++	+	++	+	6
<i>Nanopolystoma</i> Du Preez, Wilkinson et Huyse, 2008	caecilian	+	0	-	+	0	+	7
<i>Pseudopolystoma</i> Yamaguti, 1963	urodele	+	0	-	0	0	?	8
<i>Concinnocotyla</i> Pichelin, Whittington et Pearson, 1991	dipnoan	+	0	-	0	0	0	9
<i>Oculotrema</i> Stunkard, 1924	mammal	+	+	+	0	0	0	10

Position of ovary relative to worm body length: + anterior third; ++ mid body; +++ posterior. Development/length of uterus: 0 uterus absent; + uterus short, in anterior of body; ++ uterus long, occupying around half of the body anteriorly; +++ uterus very long, extending through most of the intercaecal body space. Course of uterus relative to position of ovary: + uterus restricted to space anterior to ovary, sometimes coiled but not extending into postovarian space; ++ uterus typically comprising descending and ascending loops, initially extending posterior to ovary into hindbody, then returning to forebody. Vaginae: 0 absent; + present. Development of encapsulated larvae within the body of the parent worm: 0 not recorded in literature; + rare or occasional occurrence; ++ frequent or universal mode of reproduction. Number of spines in genital coronet. In representatives infecting dipnoan and mammal hosts and in one species infecting a chelonian*: 0 penis unarmed. In amphibians, there is limited variation in the spine number characteristic for a given genus/species: + typically 5–20. ? number not known. In chelonians, spine numbers may be variable within and between species/genera: ++ typically 10–30 (species range 8–36); +++ typically 20–40 (range 2–49) but exceptionally > 100 in two species (*Polystomoides multifalx* Stunkard, 1924 and *P. stunkardi* Harwood, 1931 see Table 2 and text); ++++ typically 30–70 (range 12–97); +++++ > 100 (range 108–132). Relatively wide variations in spine number and length in polystomoidines (examples in Table 2) are evident even between representatives with limited differences in body length; in some species, spines are of two sizes alternating around the coronet. References: 1 – this account and Tinsley and Tinsley (2016); 2 – Tinsley (2004); 3 – Euzet and Combes (1964); 4 – Raharivololoniaina et al. (2011); 5 – Tinsley (1978a); 6 – Tinsley (1999); 7 – Du Preez et al. (2008); 8 – Yamaguti (1963); 9 – Pichelin et al. (1991); 10 – Tinsley (2013).

description records 108–132 (mean 123) spines; length 93–106 µm (mean 101 µm). The spines are all of a single size (in contrast to some polystomoidines that have alternating longer and shorter spines). The form of this massive copulatory organ complements the large size of the vaginal prominences: it is possible that the lobes surrounding the vaginal opening grip the penis during copulation and are impaled by the genital spines.

Taxonomy

Family: **Polystomatidae** Gamble, 1896

Subfamily: **Polystomoidinae** Yamaguti, 1963, amended Pichelin 1995

Subfamily diagnosis following Pichelin (1995) with further amendment of the following characters: uterus absent or present; egg single, remaining within oötype until released, or multiple, held in a sac-like uterus. The character “oötype short” removed. (Oötype size is equivalent to egg capsule size [and *vice versa*]; thus, egg size in the eponymous *Polystomoides megaovum* Ozaki, 1936 reaches 0.4 mm × 0.32 mm in worms up to 2.1 mm body length – Ozaki 1936: i.e. oötype length in this case is nearly 20% of body length.)

Genus: ***Uteropolystomoides* gen. n.**

ZooBank number for genus:

un:lsid:zoobank.org:act:31B4B033-DA71-4135-8793-A94CC4C23CFE

Description. Polystomatidae. Polystomoidinae. Body lanceolate. Haptor with 3 pairs of suckers of type 2 morphology (following Pichelin 1995), each with an elaborate internal skeleton of sclerites. Two pairs of hamuli, lengths of larger outer hamuli less than sucker diameter. Mouth subterminal with false oral sucker and bucco-oesophageal canal. Pharynx large, muscular. Intestinal caeca paired, extending length of body laterally, not entering haptor, without transverse diverticula, not confluent posteriorly; gut contents without dark pigment. Testis single, compact, in mid-body. Genital atrium median, opening ventrally posterior to intestinal bifurcation. Genital bulb massive with coronet of a large number (typically approaching or exceeding 100) of very long spines: spines of a single length (typically approaching or exceeding 100 µm). Ovary anterior to testis, lateral to mid-line. Vitelline follicles extending laterally along gut caeca, confluent in mid-body. Vaginae present, openings situated laterally on very large swollen prominences supporting inwardly-directed lobes. Uterus present, sac-like, containing multiple eggs with-

out appendages. Parasitic in oral and pharyngeal tracts of freshwater chelonians.

Type and only species: *Uteropolystomoides nelsoni* (Du Preez et Van Rooyen, 2015) comb. n.

Etymology: The genus name refers to the presence of a uterus which is, so far, unique amongst all Polystomoidinae.

Key to genera within the subfamily Polystomoidinae

The following alternative keys either distinguish *Uteropolystomoides* gen. n. at the first dichotomy, recognising its immediate distinction from all other genera, or indicate its parallel distinction along with *Polystomoides* from other polystomoidines followed by separation of *Uteropolystomoides* and *Polystomoides* at the last dichotomy.

- | | | |
|--|--|---|
| 1 Uterus present | <i>Uteropolystomoides</i> gen. n. | |
| – Uterus absent | | 2 |
| 2 Haptor without hamuli | <i>Neopolystoma</i> Price, 1939 | |
| – Haptor with 1 or 2 pairs of hamuli | | 3 |
| 3 Haptor with 1 pair of hamuli | | |
| | <i>Polystomoidella</i> Price, 1939 | |
| – Haptor with 2 pairs of hamuli | | 4 |
| 4 Larger hamuli with length greater than sucker diameter | <i>Uteropolystomoides</i> Tinsley et Tinsley, 2016 | |
| – Larger hamuli with length less than sucker diameter ... | | |
| | <i>Polystomoides</i> Ward, 1917 | |
| 1 Haptor without hamuli | <i>Neopolystoma</i> | |
| – Haptor with 1 or 2 pairs of hamuli | | 2 |
| 2 Haptor with 1 pair of hamuli | <i>Polystomoidella</i> | |
| – Haptor with 2 pairs of hamuli | | 3 |
| 3 Larger hamuli with length greater than sucker diameter | <i>Uteropolystomoides</i> | |
| – Larger hamuli with length less than sucker diameter ... | | |
| | | 4 |
| 4 Uterus absent | <i>Polystomoides</i> | |
| – Uterus present | <i>Uteropolystomoides</i> | |

Differential diagnosis

Du Preez and Van Rooyen (2015) reported that *Polystomoides nelsoni* (now *Uteropolystomoides nelsoni*) “is distinguished from known species by a combination of characteristics including marginal hooklet morphology, body length and haptor dimensions”. Marginal hooklets are not mentioned again after this statement in the Abstract apart from citation of their lengths in the description. Body length is contrasted with that of eight other species of *Polystomoides*, but the size range given for *U. nelsoni* actually overlaps with those cited by Du Preez and Van Rooyen (2015) for five of these species. *Uteropolystomoides nelsoni*

was also said to differ from five species (all Australasian) in length and width of the haptor, but the measurements cited for three of these actually overlap with the ranges given for *U. nelsoni* (including means for *P. australiensis* Rohde et Pearson, 1980 that are almost identical).

In their Discussion, Du Preez and Van Rooyen (2015) distinguished *U. nelsoni* from a selection of other taxa on the basis of genital spine number, but they noted that the range for *U. nelsoni* (108–132) overlaps with two other North American species, *P. multifalx* Stunkard, 1924 (120–124) and *P. stunkardi* Harwood, 1931 (92–109). The genital spines of *P. multifalx* have an unusual morphology (see Stunkard 1924) but the spines of *U. nelsoni* were not illustrated for comparison. There was no further discussion of differences between *U. nelsoni*, *P. multifalx* and *P. stunkardi*. However, the original descriptions indicate that almost all reported characteristics are closely comparable in these species and there is particularly strong similarity between *U. nelsoni* and *P. multifalx*. For these latter two species, the metrics for body length and width, haptor sucker diameter, and testis, genital bulb and egg size all overlap. Marginal hooklet length is 25–30 µm for *U. nelsoni* and 30 µm for *P. multifalx* and the respective diagrams of hooklet morphology do not indicate obvious differences.

The description of the vaginae of *P. multifalx* given by Stunkard (1924) suggests a similar structure to that of *U. nelsoni*: “the vaginae are wide and there (*sic*) walls are so folded that in whole mounts they may seem to have several openings”. Stunkard’s (1924) illustration shows a clear space around the openings of the vaginae from which the vitelline follicles, otherwise widely distributed throughout the body, are apparently displaced. Although vaginae were not included in Du Preez and Van Rooyen’s (2015) account of *U. nelsoni*, their holotype drawing and the paratypes examined in this present study illustrate the exceptional size of the swollen tissue surrounding the vaginal openings. Stunkard (1924) also emphasised the large size of the genital bulb (termed a ‘cirrus sac’) in *P. multifalx* and the lengths cited for both species overlap. Indeed, Stunkard (1924) observed that genital bulb size and genital spine number were distinctive features of *P. multifalx*, concluding that the number of spines was three times greater than in any other then-known polystome. With the greater number of species now described, this distinction is still evident for *P. multifalx*, *U. nelsoni* and *P. stunkardi* (Table 2).

Some other comparisons are less clear-cut: Stunkard (1924) cited several measurements without precision (e.g. larger and smaller hamuli reported as 0.2 and 0.1 mm respectively), and Du Preez and Van Rooyen (2015) reported some metrics with unusually large ranges (e.g. haptor sucker diameter from 148 to 781 µm) perhaps suggesting wide age variation. Further interpretation of potential differences is limited by small sample size (*P. multifalx* described from two specimens and *U. nelsoni* from ten). Adding to difficulties of species’ comparisons, the identity of the host of *P. multifalx* may be uncertain. The two parasite species were reported to infect related hosts, *Pseudemys*

Table 2. Summary of genital spine characteristics in Polystomoidinae.

Taxa	Spine number	Sample size	Spine length (µm)
	Range	Individuals reported	Range
<i>Polystomoides</i> -like species in southeastern USA			
<i>Uteropolystomoides nelsoni</i> (Du Preez et Van Rooyen, 2015)	108–132	10	96–106
<i>Polystomoides multifalx</i> Stunkard, 1924	120–124	2	
<i>Polystomoides stunkardi</i> Harwood, 1931	92–109 ¹ ; 82–130 ²	8; 25 (approx.)	> 80 (see text)
Other <i>Polystomoides</i> spp.	Range for species with greatest number	Species considered	Range for species with greatest length
North America	36–49	4	57–64
South America	29–35	5	56
Rest of world	42–47	7	75–88
<i>Uropolystomoides</i> in Australasia	Range	Individuals	Range
<i>U. australiensis</i> (Rohde et Pearson, 1980)	67–95 ³ ; 74–97 ⁴	4; 6/17 ⁵	78–106; 86–110
<i>U. malayi</i> (Rohde, 1963)	68–83	25	81–102
<i>U. scottae</i> (Pichelin, 1995)	68–79	5/19 ⁵	54–66
Other genera worldwide	Maximum	Species considered	Maximum
<i>Uropolystomoides</i> Tinsley et Tinsley, 2016	64	10	69
<i>Neopolystoma</i> Price, 1939	36	22	48/57 ⁶
<i>Polystomoidella</i> Price, 1939	16	3	15/22 ⁶

¹ recorded by Harwood (1931); ² Price (1939); ³ Rohde and Pearson (1980); ⁴ Pichelin (1995); ⁵ sample sizes for spine number and spine length, respectively; ⁶ genital coronet with spines of two sizes; other data sources, see text.

floridana (LeConte) and *Pseudemys nelsoni*, in the same geographical area (Florida, USA) but the latter turtle, described in 1938, was not distinguished as a separate species at the time of Stunkard's (1924) studies on *P. multifalx* in *P. floridana*. Current assessments recognise the close relationship of these host species which may co-exist in the same habitats (e.g. Jackson 2010).

The importance of host identity in polystomoidine species separation is unclear: while strict host specificity is a significant feature of many polystomatids infecting anuran amphibians, the host specificity of chelonian polystomatids may be less strict (see Pichelin 1995, Tinsley and Tinsley 2016). Nevertheless, without voucher specimens of the hosts found infected, it will be difficult to resolve uncertainties of host identity: large population samples of hosts and parasites, identified by molecular characters, are required. In summary, considering the full range of characteristics reported in the original descriptions, there is no convincing separation of *P. multifalx* and *U. nelsoni* – except for the presence of a uterus in the latter.

Comparisons involving *P. stunkardi* are less conclusive. Du Preez and Van Rooyen (2015) commented only that this species has a large number of genital spines but did not discuss how it and *P. nelsoni* may be distinguished. The validity of *P. stunkardi* has been disputed. Price (1939) argued for the synonymy of *P. stunkardi* with *P. multifalx*. Harwood (1931) separated *P. stunkardi* by its smaller number of genital spines (based on specimens from Oklahoma), but Price (1939) reported specimens from Florida with 82–130 spines overlapping the ranges for both *P. stunkardi* and *P. multifalx*. However, Price was in error in citing a range of 100–124 spines for *P. multifalx*: the two specimens recorded by Stunkard (1924) had 120 and 124 spines. Subsequent reviewers have endorsed this synonymy (in-

cluding Sproston 1946, Yamaguti 1963, Morrison and Du Preez 2011) although Du Preez and Van Rooyen (2015) referred to *P. stunkardi* as a distinct species.

However, the original descriptions include one potentially significant feature not discussed by later authors: marginal hooklet length was said to be 'about 20 µm' in *P. stunkardi* (see Harwood 1931) but 30 µm in *P. multifalx* (see Stunkard 1924). The scale of this difference would be unusual for conspecific polystomatids so, if correct, the two taxa may indeed be distinct. Other characteristics that would guide comparison of *P. stunkardi* with *U. nelsoni* (and *P. multifalx*) are uncertain from the account of Harwood (1931). Genital spine length (80 µm) was based on a sectioned specimen and may be underestimated. The ranges of several measurements overlap but these may be less informative given the wide size variation cited for both *P. stunkardi* and *U. nelsoni* and the possibility that both studies include small growth stages.

There is no information (or an illustration) for the morphology of the hamuli of *P. stunkardi* and the drawings given by Stunkard for *P. multifalx* lack detail (although they appear to show slender spindle-like hamuli, distinct from the subdivided roots recorded for *U. nelsoni*). The vaginae of *P. stunkardi* were not described but Harwood's diagram depicts the openings as large lacunae. Their size is emphasised by the representation of the vitelline follicles which are extensive elsewhere but excluded from the area surrounding the vaginal openings (as in *P. multifalx*). This large structure complements the equally large genital bulb, described by Harwood (1931) as having a "genital coronet very similar to that of *P. multifalx*". No eggs were reported in the eight specimens examined by Harwood (1931) and the organisation of the distal ducts of the female tract is unknown; so, conclusive comparison with the major character of *U. nelsoni* – presence of a uterus – is precluded.

The differentiation of *U. nelsoni* by Du Preez and Van Rooyen (2015) made no reference to other North American species of *Polystomoides*, but there is clear separation on the basis of genital spine number and size from *P. coronatus* (Leidy, 1888) (the type species), *P. oris* Paul, 1938, *P. pauli* Timmers et Lewis, 1979 and the various species presumed by Price (1939) to be synonyms of *P. coronatus* (synonymy that is probably unjustified: see Tinsley and Tinsley 2016). The published descriptions of North American species of *Polystomoides* show that *U. nelsoni*, *P. multifalx* and *P. stunkardi* are the only species with spine number exceeding 100 (maxima 132, 124 and 130, respectively): records for all other species are less than 50 spines (Table 2). The exceptional length of these genital spines in *U. nelsoni* (maximum 106 µm) also shows clear separation from other North American species (with maximum 64 µm in *P. pauli*). Even though the genital spine length for *P. stunkardi* may be underestimated from sectioned material, Harwood's measurement (80 µm) is still much greater than the maximum in other North American species of *Polystomoides*. Morrison and Du Preez (2011) recorded a genital spine length of 80 µm for *P. albicollis* MacCallum, 1919 but the original description actually specified an improbable '.80 µ'; Stunkard (1924) noted much confusion in MacCallum's measurements, so all these data require re-evaluation. Genital spine characteristics provide unambiguous separation of *U. nelsoni* from all South American species of *Polystomoides* (maximum spine number 35 and maximum length 56 µm) (Table 2).

In wider comparisons across the worldwide distribution of polystomoidines, there are only three species that have genital spine number and length approaching those for *U. nelsoni* (Table 2). A series of studies by Rohde (1965), Rohde and Pearson (1980) and Pichelin (1995) provide ranges for *Uropolystomoides australiensis* (Rohde et Pearson, 1980) of 67–97 spines (mean length 97 µm), *U. malayi* (Rohde, 1963): 68–83 spines (mean length 94 µm), and *U. scottae* (Pichelin, 1995): 68–79 spines (mean length 60 µm). The respective drawings show relatively large vaginal openings but these are sublateral, nearly overlying the intestinal caeca on each side, not forming the prominent lateral swellings on the body margin characteristic of *U. nelsoni*. However, these three Australasian species are all urinary tract parasites, phylogenetically distinct from the North American oral cavity species of *Polystomoides*. Data available for other polystomoidine species record smaller genital spine numbers: maxima <65 in other species of the genus *Uropolystomoides* Tinsley et Tinsley, 2016, <50 in spp. of *Polystomoides*, <40 in spp. of *Neopolystoma* Price, 1939, <20 in spp. of *Polystomoidella* Price, 1939 (Table 2).

These comparisons suggest first, that *U. nelsoni*, *P. multifalx* and *P. stunkardi* form a coherent group of apparently-related species and, second, that there is a major discontinuity between these and all other polystomoidines worldwide. However, whatever emphasis is given to the differences in copulatory structures (genital bulb, spines and vagina), the most fundamental distinguishing characteristic of *U. nelsoni*, not discussed in the original descrip-

tion, is the presence of a uterus. If this characteristic is not considered, there appear to be no features reported in the respective accounts of Stunkard (1924) and Du Preez and Van Rooyen (2015) justifying separation of *P. multifalx* and *U. nelsoni*. However, with inclusion of the uterus in systematic assessment, the features of *U. nelsoni* are unique amongst chelonian polystomatids (Table 1) and have major significance for reproductive biology and for interpretation of evolutionary relationships.

DISCUSSION

Terminology

The terminology of the distal reproductive tract of the Polystomoidinae has been confused for 100 years despite various corrections. The distinctiveness of polystomatids infecting chelonians was recognised by Ward (1917) noting a “short uterus containing only a single egg”. Stunkard (1917) employed the term ‘uterus’ for the chamber that contains the single egg and applied the name ‘oötype’ to the duct that receives the vitello-vaginal ducts and the oviduct. This usage for ‘uterus’ was also followed in other descriptions of polystomoidine species (e.g. MacCallum 1919, Harwood 1931). In a few cases, authors have used ‘uterus’ to describe the very short duct that links the oötype to the genital atrium, but this cannot be considered equivalent to the uterus of other flatworms: it is too small to hold an egg capsule *en route* from the oötype to the outside. Morrison and Du Preez (2011) gave a schematic diagram of the reproductive organs of chelonian polystomatids showing both a uterus and an oötype, but the labelling is in error since the ‘uterus’ is shown proximal rather than distal to the oötype (the structure labelled is actually the ovovitelline duct).

The major systematic revision of Price (1939) included a definition for chelonian polystomatids that was adopted by several subsequent reviewers: “uterus short, usually containing one egg at a time” (e.g. Sproston 1946, Yamaguti 1963, also Ozaki 1935 but, in this latter case, without ‘usually’). The qualification ‘usually’ is not supported by the literature: none of the taxonomic descriptions of the polystomoidine species refers to the presence of more than one egg. Some authors reporting large population samples of particular species are explicit on this point, for instance Rohde (1965): “...never contains more than one egg”. The review by Morrison and Du Preez (2011) appears to provide one exception. Their data listing number of ‘intra-uterine eggs’ for all chelonian polystomatids confirm that, where originally recorded, the species have only a single egg except in the case of *Neopolystoma elizabethae* Platt, 2000 which is listed as having up to three eggs. However, the original description of this species recorded “n=3” as the sample size for the number of eggs measured not for the number present in an individual worm (see Platt 2000).

The correct interpretation of the structure containing the egg capsule was provided *inter alia* by Paul (1938), Bychowsky (1957) and Williams (1961, 1995): each emphasised that the term uterus had been incorrectly applied to this chamber, that this is strictly the oötype, and that a true

uterus is absent. The character “uterus absent, all species” was included by Pichelin (1995) in her amended diagnosis of the subfamily Polystomoidinae.

Functional significance of the uterus

Across the Polystomatidae, the uterus exhibits major variations in size, development and function corresponding with the diversity of life cycle patterns. Representatives infecting anuran amphibians exploit the characteristic that almost all anurans, even those with highly terrestrial life styles, must return to water to breed. Indeed, where the host's ‘aquatic phase’ is briefest the parasite reproductive modifications, especially to the uterus, are most specialised (Tinsley 1993). Thus, exceptionally amongst all platyhelminths, the uterus of *Pseudodiplorchis americanus* is an active synthetic structure that delivers nutrients continuously to stored larvae; the uterine wall produces stacks of membranes that are added to the flexible ‘egg shell’ enabling it to increase in size as the enclosed embryo grows (Cable and Tinsley 1991). These and other adaptations of the uterus are major evolutionary innovations over the ‘inert tube’ typical of some monogeneans (reviewed by Kearn 1986, Cable et al. 1998, Tinsley 2004). Tinsley (1983) considered that an elongated uterus evolved independently in several polystomatid lineages infecting anurans: the separate pathways towards hyper-development of the uterus are reflected in different arrangements of the ovary, testis, vitellaria, vaginae and gut (see also Table 1).

In contrast, there are nine genera of polystomatids in which a uterus is absent: *Protopolystoma* Bychowsky, 1957 (in an anuran host), *Nanopolystoma* Du Preez, Wilkinson et Huyse, 2008 (in a caecilian), *Pseudopolystoma* Yamaguti, 1963 and *Sphyrnura* Wright, 1879 (in urodeles), *Concinnocotyla* Pichelin, Whittington et Pearson, 1991 (in a lungfish) and *Polystomoides*, *Uropolystomoides*, *Polystomoidella* and *Neopolystoma* (in chelonians) (Table 1). Their hosts share the characteristic that they are completely (or predominantly) aquatic. Eggs produced by these parasites are likely to be deposited directly into the environment where transmission can occur. In those cases where information exists, eggs assembled in the oötype are released in continuous succession: there is no role for a uterus as a storage organ as in other polystomatids.

Possession of a uterus by *U. nelsoni* could give several potential advantages for reproductive biology. In the other known representatives of the Polystomoidinae, and in other polystomatids without a uterus such as *Protopolystoma*, the tanning of each egg is completed in the oötype before expulsion (Tinsley and Owen 1975). The absence of a uterus has the consequence that hardening of the egg capsule must be completed before production of the next egg can begin. The few studies reported for chelonian polystomatids indicate low rates of egg production: for instance, output of 2–3 eggs/worm/day (e/w/d) for a species of *Polystomoides* (Paul 1938); a range 0.7–5.6 e/w/d for one species of *Polystomoides* and two species of *Neopolystoma* (Pichelin 1995); and 1.5 e/w/d for a species of *Neopolystoma* (Du Preez and Lim 2000). These rates suggest that the total time for production of each egg (including

any interval when the oötype is empty) may range from about 4 h to more than 24 h. So, the presence of a uterus in *U. nelsoni* – as in other polystomatids with a uterus – could increase slow egg formation rates by providing a site in which capsule hardening can be completed without blocking assembly of the next egg (reviewed by Tinsley 1983).

Temporary storage of eggs by the uterus could also provide an advantage in transmission success if egg capsules are retained by the parent worm during periods when release would invariably lead to loss – as would occur when a semi-aquatic host emerges onto land. Kearn (1986) suggested that the uterus of *Oculotrema hippopotami* Stunkard, 1924 may perform this role if eggs produced during the night are retained *in utero*, when the host forages on land, and are then released during the day when hosts are submerged in water. For polystomoidine species in the oral cavity and eye of chelonians, eggs released continuously from the oötype are likely to accumulate in mucus when the host leaves water and are then washed from the infection site when the host returns to water. It is not known whether this affects egg survival but, in similar circumstances, the uterus of *U. nelsoni* would provide direct protection for eggs stored within the parent worm, especially if the duration of host desiccation is prolonged (for instance, during seasonal dry periods).

The uterus could have another important role in reproductive biology if eggs are retained within the parent for periods allowing the larvae to complete development (Table 1). Incubation within the parent worm can reduce or eliminate the hazards and time delays of egg development in the external environment (Tinsley 1983). These advantages are seen in a series of polystomatids infecting anuran amphibians where larvae that have completed development remain within their egg capsules in the uterus and hatch immediately after discharge into water (Tinsley 1978b). Further development of this trait involving retention of eggs within the parent may facilitate an internal cycle of autoinfection if the larvae hatch *in situ*. This may involve only one or a few eggs within the uterus (as sometimes occurs in species of *Polystoma* Zeder, 1800 – see Combes 1967, Tinsley 1983), but each larva hatching without leaving the host individual can instantly boost existing worm burdens at every ‘cycle’.

The same phenomenon may occur in polystomoidine species without a uterus: several studies have recorded a fully-developed oncomiracidium within an egg capsule retained in the oötype of African species of *Uropolystomoides* (Euzet and Combes 1965, Tinsley 1973, Kulo 1980). Remarkably, those larvae completing development within the parent lack tegumental cilia and sense organs involved in host invasion indicating that the developmental pathway towards autoinfection is pre-programmed in the oötype (Lambert and Kulo 1982). The highest level of ovoviviparity in polystomatids is seen in species of *Polystomoidella* where the larva completes development inside its capsule held in the oötype and then hatches and continues to grow while still inside the parent worm (Oglesby 1961). These life cycle innovations may occur in polystomatids both with and without a uterus (Table 1), but the numerical

effect of an internal cycle of autoinfection is correspondingly greater where the parent is equipped to retain multiple eggs *in utero*, as shown in *Eupolystoma alluaudi* (de Beauchamp, 1913) by Fournier and Combes (1979).

In polystomatids without a uterus, development of the egg within the oötype has the inevitable cost that further egg assembly is halted. Where the larva completes development to the point of hatching, this period of reproductive arrest is likely to last several weeks (discussed by Tinsley 1983). On the other hand, in polystomatids with a uterus, egg production may continue without interruption in the oötype alongside accumulation of eggs *in utero* that may complete development, giving the potential either for release and external host-to-host transmission or for a direct internal cycle of re-infection.

These cases provide precedents for the potential advantages of a uterus in *U. nelsoni*, but there is insufficient information to extrapolate further in this unique example. Du Preez and Van Rooyen (2015) commented that all eggs in their sample of *U. nelsoni* were undeveloped but the possibility of retention and *in utero* development seasonally during specific periods of host activity (and inactivity) requires further investigation.

Significance of the uterus for evolutionary biology

The distinguishing feature of polystomoidines – that the four previously-known genera have the same simplified egg assembly apparatus lacking a uterus – is important for interpreting evolutionary relations. This uniformity is counter-intuitive. The range of chelonian hosts exhibits significant variation in ecology and behaviour: hosts typically have a close association with water but periods out of water may include episodes of basking lasting hours and of aestivation/hibernation lasting weeks or months. It might therefore be expected that this ecological diversity would be reflected in differences in parasite reproductive specialisations and life cycle patterns. However, although the uterus has a major role in transforming the life cycles of anuran polystomatids, its potential is entirely missing in polystomoidines (until the discovery of *U. nelsoni*). Standardisation of reproductive morphology in *Polystomoides*, *Uropolystomoides*, *Neopolystoma* and *Polystomoidella* is also surprising because their lineages have a more ancient evolutionary history than the highly diverse taxa infecting anurans (Héritier et al. 2015) (Table 1). This suggests that the uterus was lost in the distant ancestor(s) of all four genera and has not been ‘regained’ even when host ecology might suggest a considerable selective advantage for a reproductive mode employing a uterus. So, the existence of a chelonian polystomatid with a uterus containing multiple eggs indicates a major innovation in polystomoidine life cycles.

There is an important precedent amongst polystomatids for the expression of genes controlling alternative designs of reproductive tract – with and without a uterus. In species of *Polystoma*, ontogeny follows one of two pathways leading either to accelerated development of precocious adults without a uterus (so-called ‘neotenic’) or to slowly-growing ‘normal’ adults with a uterus (reviewed in

many general textbooks of parasitology including Kearn 1998). The genetic architecture for both modes must be present simultaneously in the genome of all individuals of *Polystoma* spp. In chelonian polystomatids, the equivalent genetic control of reproductive development may be separated at the level of different lineages. So, it might be conjectured that *U. nelsoni* has retained (or reactivated) the genes controlling development of a functional uterus while these are absent or entirely suppressed in all other known polystomoidines.

Williams (1995) considered that the polystomoidines (and other polystomatids lacking a uterus, including *Protopolystoma* and *Sphyrnura*) are neotenic forms. This interpretation would concur with the view that the presence of a uterus represents the ancestral condition in polystomatids. Loss of the uterus might have occurred independently in each of the four ‘without uterus’ lineages of polystomoidines, but their equivalent morphological organisation suggests that loss occurred in their common ancestor, pre-dating their divergence. This event could be older (perhaps very much older) than 150 Mya according to the evidence of plate tectonics, biogeography and molecular biology (see Rohde and Pearson 1980, Verneau et al. 2011, Héritier et al. 2015). Then, the presence of a uterus in *U. nelsoni* could be explained by alternative scenarios, including: (i) that the uterus has developed *de novo* in a *Polystomoides*-like lineage (under selection pressure from host ecology) without direct genetic links to the structure lost in the early history of the other polystomoidines; (ii) that evolution involved an atavism from the closely-similar *Polystomoides* lineage, with reactivation of suppressed/inactive genes; (iii) that the uterus has been present throughout the evolutionary history of the *Uteropolystomoides* lineage, derived directly from a ‘with uterus’ ancestor.

If the uterus is a new evolutionary trait, its appearance would be remarkable, presumably involving the coordinated development of a complex structure requiring novel genetic architecture. The possibility of an atavistic origin with reversion to the original ancestral condition – with a uterus – would require reactivation of a genetic programme that may have been suppressed for many millions of years. Both of these origins would have involved a predecessor in which the uterus had been lost. The third possibility, that the uterus has existed continuously in a ‘with uterus’ lineage, presumes the oldest origin and an independent evolutionary pathway for *Uteropolystomoides* separated from other known polystomoidines throughout the phylogeny of the subfamily. For each of these hypotheses, the distinctive morphology of *Uteropolystomoides* could support the assumption that the genus represents an ancient evolutionary line. Evidence for the long isolation of this lineage is provided by molecular phylogenetic data showing *U. nelsoni* basal to other Nearctic Polystomoidinae (Verneau et al. 2011, Du Preez and Van Rooyen 2015) (although not basal to Old World lineages).

The evolutionary and developmental mechanisms are likely to be far more complex than suggested by these speculative scenarios but they illustrate the rich possibilities of phylogenetic relationships in an ancient parasite

group with a history that extends over enormous periods of time, during the drifting apart of the Gondwana continents (see the phylogeny of Héritier et al. 2015). It may therefore be relevant to these mechanisms that Littlewood et al. (1997) calculated particularly slow molecular evolutionary rates in chelonian polystomatids. Indeed, this is a host-parasite association in which morphological evolution appears to have been very slow for both the hosts and the parasites.

Significance of the uterus for the systematics of the Polystomoidinae

Until this present account, major emphasis in morphological systematics of the Polystomoidinae has focused on simple differentiation employing haptor attachment organs (see taxonomic keys, above). *Uteropolystomoides nelsoni* is clearly distinguished from species of *Neopolystoma* (without hamuli) and from *Polystomoidella* (with one pair of hamuli); it is aligned on this basis with *Polystomoides* (*sensu* Tinsley and Tinsley 2016) with two pairs of hamuli that are small relative to sucker diameter and infect oral cavity sites, distinct from *Uropolystomoides* whose species have two pairs of hamuli that are large relative to the suckers and infect the urinary tract (Tinsley and Tinsley 2016). Recognition of *Uteropolystomoides* introduces another fundamental character into assessment of systematic relationships: the distinguishing feature of this genus (and the single species so far included) has no precedent elsewhere in the subfamily. Whilst the presence of a uterus is unique, *U. nelsoni* appears to have a close morphological affinity with *P. multifalx* (and with *P. stunkardi* if this is a distinct species). Indeed, although some relevant details are incomplete, *U. nelsoni* and *P. multifalx* cannot reliably be distinguished morphologically except for the presence/absence of a uterus. This raises the possibility that *U. nelsoni* is an aberrant form of *P. multifalx* – a developmental freak. If so, the occurrence of a uterus in this single species would be of exceptional significance for developmental biology and the control of organogenesis. Inclusion of the species *U. nelsoni* within the genus *Polystomoides* would require amendment of the generic diagnosis to include ‘uterus almost always absent, exceptionally present with multiple eggs *in utero*’. However, the presence of a uterus in *U. nelsoni* is a fundamental innovation, representing the addition of a complex structure unknown in the global distribution of the subfamily. Indeed, the structure of the uterus – as a sac rather than a tubular duct – is atypical across the wider Polystomatidae. It might be counter-intuitive for a systematic subdivision at the level of genus to include representatives with and without a structure of such biological importance. It seems more likely that this trait indicates the existence of an independent lineage within the Polystomoidinae.

There is an alternative possibility which, with the current incomplete knowledge of morphology, must remain conjectural. The original description of *P. multifalx* (as *Polystoma multifalx*) was based on only two specimens, each containing a single egg capsule (Stunkard 1924). There have been no subsequent morphological descriptions. The type collection of *U. nelsoni* shows that three of the nine ovigerous specimens contain a single egg, so the two specimens studied by Stunkard could have been at the same stage of egg production and accumulation as these three individuals. The single account of *P. stunkardi*, based on eight specimens, does not mention eggs and none is shown in the illustration by Harwood (1931), so the worms may have been young adults, not yet egg producing. For both, it cannot be excluded that a uterus might be present.

Despite the limitations of the original descriptions, the trio of species *P. multifalx*, *U. nelsoni* and *P. stunkardi* comprise a group characterised by the large size of their copulatory structures. The species descriptions and diagrams suggest common development of a large genital bulb, large number of genital spines, large size of the spines and, correspondingly, hyper-development of the vaginal apertures. These features set the three taxa apart not only from *Polystomoides* but also from all other American species and all polystomoidine species in the oral tract of chelonians worldwide. The characters do not form part of a continuum of states and sizes extending the variation evident in other species: instead, the respective ranges are separated by clear discontinuities (Table 2).

The close morphological similarities within this trio of taxa are reinforced by their infection of related hosts, species of *Pseudemys* Gray, in the same geographical region. So, there would be justification to assign these three species to a distinct genus based on their synapomorphic copulatory structures. However, it is not inconceivable that *P. multifalx* and *P. stunkardi* may actually also share the presence of a uterus with *U. nelsoni*. If future studies were to confirm this working hypothesis then *P. multifalx* and *P. stunkardi* should be re-assigned to the genus *Uteropolystomoides*, but, in the present state of knowledge, systematic revisions should be cautious. The major conclusive feature to emerge from these comparisons is that the species *U. nelsoni* has a character that is, so far, unique amongst polystomoidines and this provides the strongest basis for recognition of a distinct genus. While there is convincing evidence for a close morphological relationship with *P. multifalx* and *P. stunkardi*, assessment of their systematic status must await further studies and, at present, the genus *Uteropolystomoides* remains monotypic.

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